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SHORT COMMUNICATION

Gene Expression by Polymeric-TAR Decoys

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The *tat* gene product (Tat) of human immunodeficiency virus type 1 (HIV-1) is an early regulatory protein which transactivates HIV-1 gene expression by interacting with the *trans*-activation response element (TAR) present in the HIV-1 long terminal repeat (LTR). In HIV-1-infected cells Tat can also activate the expression of tumor necrosis factor (TNF). Recent results indicate that essential for this effect is the interaction of Tat with a TAR-like structure present in the TNFβ messenger RNA leader region that closely resembles the TAR of the HIV-LTR. Here we show that because of this similarity of mechanisms, the expression of an RNA species encoding polymeric-TAR sequences and known to inhibit Tat-mediated HIV-1 gene expression also blocks TNF gene expression in response to Tat, but not TNF promoter activation induced by human T cell leukemia/lymphotropic virus type I Tax protein. Since TNF is increased in HIV-1-infected individuals and can activate HIV-1 gene expression or rescue Tat-defective HIV-1 proviruses, activation of TNF by Tat may be part of a complex pathway in which HIV-1 uses its own expression to increase infectivity and to induce disease. This study shows a dual role for the polymeric-TAR construct in inhibiting HIV-1 replication and strengthens the potential use of this protective gene in gene therapy for AIDS.

The human immunodeficiency virus type 1 (HIV-1) encodes an early regulatory protein called Tat (*1, 2*). Tat transactivates HIV-1 gene expression by interacting with the Tat-activation response element (TAR) in the HIV long terminal repeat (LTR) (*3–7*). Tat promotes transcriptional initiation of the integrated proviral genome and stimulates the elongation of newly initiated viral transcripts (*8–10*). Tat has also been shown to be released by infected T cells (*11–13*). In this extracellular form Tat activates HIV replication and rescues Tat-defective proviruses in a paracrine fashion (*10–12, 14–18*). By a different mechanism (receptor-mediated) extracellular Tat participates in the development and progression of Kaposi's sarcoma (KS) (*11, 12, 14, 19–23*). In addition, recent data indicate that Tat is involved in the T cell death occurring in AIDS by inducing or contributing to apoptosis (*13, 24*). In HIV-1-infected or *tat*-transfected cells or when extracellular Tat is present at high concentrations, Tat can also activate the expression of several cellular genes including tumor necrosis factor (TNF) α and β (*25–27*), transforming growth factor β (*28*) and others according to the cell type (reviewed in Ref. *29*). In particular, TNF is increased in HIV-1-infected individuals (*30, 31*) and is known to affect the vascular, immune, and central nervous systems, suggesting a role for this cytokine in the pathogenesis of AIDS-KS, B cell lymphomas, and central nervous system disorders (*14, 25, 32–34*). In addition, TNF activates HIV-1 gene expression and rescues Tat-defective HIV-1 proviruses (*14, 35, 36*). TNF can also increase production of interleukin 1 (IL-1) and IL-6 (*25*), which are increased in HIV-1-infected individuals (*37, 38*). Thus, activation of TNF by Tat may be part of a complex pathway in which HIV-1 uses viral products and host factors to increase its own expression, infectivity, and to induce disease (*25*). Because of these effects of TNF, it is critical that any gene therapy approach be able to block the Tat-induced gene expression of TNF in addition to inhibiting HIV replication.

The TAR element is present in all HIV RNA transcripts and forms a predicted stem–loop secondary structure that is required for Tat function (*5, 6*). Expression of TAR RNA (polymeric-TAR) has been found to act as a decoy to bind and sequester Tat produced in the cells (*39–43*) or taken up by the cells (*44*), thus preventing its binding to the TAR sequence encoded by the viral RNA. This

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results in the lack of activation of viral gene expression and diminished generation of progeny virus.

Recent data indicate that Tat-induced TNFβ gene expression is mediated by a TAR-like element present in the TNFβ 5′ untranslated region although both SP1 and NFκB binding sites are also required for Tat activity (*26*). The TNFβ messenger RNA leader region, in fact, contains a sequence which is 80% homologous to the bulge of the TAR and forms a predicted stem–loop structure that closely resembles the TAR of the HIV-LTR (TAR-like). This region is essential for TNFβ transactivation by Tat (*26*). This apparent similarity of mechanisms suggested that the polymeric-TAR construct may inhibit Tat-mediated TNFβ gene expression in addition to inhibition of HIV-1 gene expression.

To test this hypothesis, cotransfection experiments were performed with $CD4^+$ T cell lines such as Jurkat cells, H9 cells permanently transduced with the first exon of Tat (H9-Tat 1) and the 8E51 cell line which is chronically infected with a *pol*-defective HIV-1 provirus (*26, 45*). These cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37° in a humidified atmosphere containing 5% carbon dioxide in air. The plasmids used for the transfection experiments included pTNFβCAT and pCD7CAT which contain the chloramphenicol acetyltransferase (CAT) reporter gene linked to the TNFβ-promoter (pTNFβCAT) or HIV-1 LTR (pCD7CAT) described previously (*26, 46*). The pCVTAT plasmid and its control vector, pCV0, have also been described (*1, 44*). The human T cell leukemia/lymphotropic virus type I (HTLV-I) Tax plasmid (*47*) and the plasmids LTR-50 TAR and the control vector LTR-0 TAR contain 50 and 0 copies, respectively, of the TAR element under the control of the HIV-1-LTR promoter (*39, 41*), have all been described. Transfection of the plasmid DNA was performed by the electroporation procedure (*25*). Briefly, cells (3×10^7) were resuspended in 0.25 ml of growth medium containing 1 m*M* EGTA and mixed gently with plasmid DNA. After 10 min incubation on ice, cells were electroporated at 960 μ Far, 0.25 kV. After an additional 10 min on ice, the cells were resuspended in 30 ml of growth medium and harvested 72 hr later. CAT assays were performed on cell lysate (normalized to total protein content) as described previously (*46*).

To test whether the polymeric-TAR construct may inhibit Tat-mediated TNFβ gene expression, 2 μg of pTNFβCAT and 2 μg of either pCVTAT or pCV0 were cotransfected with 6 μg of either LTR-0 TAR or LTR-50 TAR into Jurkat cells. The amount of polymeric-TAR DNA transfected in these experiments is about a 2-fold and a 3-fold molar excess of pTNFβCAT and pCVTAT, respectively. As shown in Fig. 1A and Table 1A, pCVTAT increased pTNFβCAT activity by 4.7-fold, as compared to the control vector pCV0, and the polymeric-TAR decoys inhibited Tat-mediated TNFβ gene expression by 4.4-fold (Table 1A) or 71% (Table 1B) and up to 78% (Fig. 1A) as

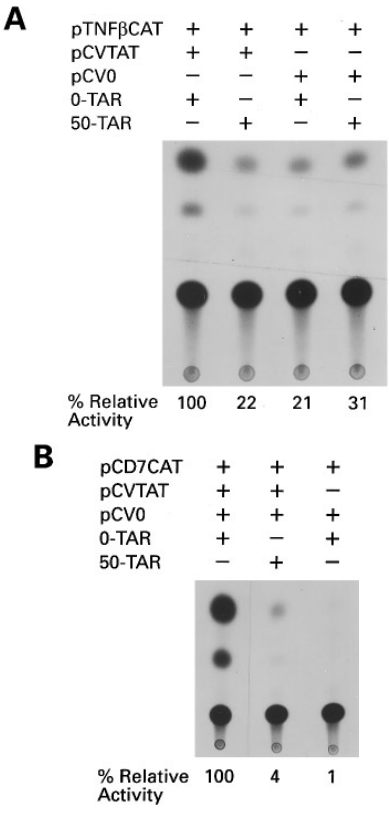

FIG. 1. Inhibition of Tat-mediated activation of the TNFβ promoter or the HIV-1 LTR by polymeric-TAR in Jurkat cells. Representative CAT assays are shown. (A) Cotransfection of pTNFβCAT (2 μg) and pCVTAT (2 μg) or pCVO (2 μg) with LTR-0 TAR (6 μg) or LTR-50 TAR (6 μg). (B) Cotransfection of pCD7CAT (1 μg) and pCVO (3 μg) with LTR-50 TAR (3 μg) or LTR-O TAR (3 μg) in the presence or absence of pCVTAT (0.1 μg). CAT activity from cells transfected with the pCD7CAT or pTNFβCAT, PCVTAT, and LTR-O TAR was given a value of 100%. The percentage of relative CAT activity is shown at the bottom.

compared to cells transfected with the control vector LTR-0 TAR, reaching almost the baseline levels observed in the absence of Tat (Fig. 1A and Table 1A). Since LTR-50 TAR and LTR-0 TAR induced the same TNFβCAT activity in the absence of pCVTAT, this suggests that LTR-50 TAR alone does not suppress TNFβ-CAT transcription. Therefore, the difference in TNFβCAT activity observed with the LTR-50 TAR and the LTR-0 TAR in the presence of pCVTAT is really dependent on the ability of LTR-50 TAR to trap the Tat protein and it is not due to the activation of other inhibitory activities such as the effect of double-stranded kinase which has been reported to be activated by TAR (*48*).

To compare the level of transactivation or inhibition of TNFβ-mediated gene expression with the HIV-1 LTR-mediated gene expression by Tat or polymeric-TAR, respectively, pCD7CAT (1 μg), pCV0 (3 μg), and either LTR-0 TAR or LTR-50 TAR (3 μg) were cotransfected in the presence or absence of pCVTAT (0.1 μg) into Jurkat cells (Fig. 1B). pCVTAT activated HIV-1 LTR-CAT (pCD7CAT) by 135-fold as compared to the control vector pCV0 and

TABLE 1A

Activation of TNFβ-CAT or HIV-1 LTR-CAT Gene Expression by Tat and Inhibition by the Poly-TAR Decoy

Plasmid DNA	Percentage of CAT activity	Activation inhibition
pTNFβCAT + pCV0 + LTR-0 TAR	1.7	
pTNFβCAT + pCVTAT + LTR-0 TAR	8	4.7*X*
pTNFβCAT + pCVTAT + LTR-50 TAR	1.8	4.4*X* (78%)
pCD7CAT + pCV0 + LTR-0 TAR	0.17	
pCD7CAT + pCV0 + pCVTAT + LTR-0 TAR	23	135.3*X*
pCD7CAT + pCV0 + pCVTAT + LTR-50 TAR	0.83	27.7*X* (96%)

Note. Jurkat cells were transfected with pTNFβCAT (2 μg), pCV0 (2 μg), or pCVTAT (2 μg) and LTR-0 TAR (6 μg) or LTR-50 TAR (6 μg); or with pCD7CAT (1 μg), pCV0 (3 μg), and LTR-50 TAR (3 μg) or LTR-0 TAR (3 μg) in the presence or absence pCVTAT (0.1 μg). CAT activity was determined as described previously (*18*). The percentage of CAT activity is shown. The numbers in parenthesis refer to the percentage of inhibition of CAT activity by poly-TAR decoys compared with that of the control vector LTR-0 TAR. *X*, fold of activation or inhibition by Tat or poly-TAR, respectively.

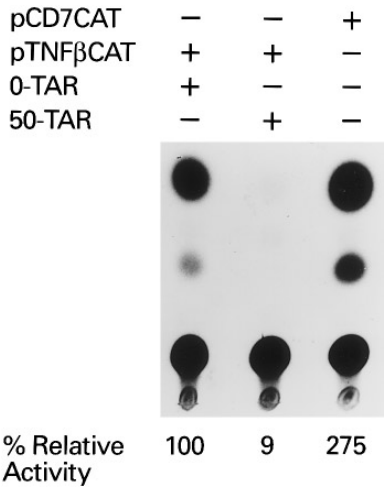

FIG. 2. Inhibition of Tat-mediated activation of the TNFβ promoter by polymeric-TAR in HIV-1 chronically infected 8E51 cell line. Shown is a representative CAT assay after transfection of pTNFβCAT (5 μg) and LTR-50 TAR (50 TAR) (5 μg) or LTR-0 TAR (0 TAR) (5 μg) into 8E51 cell line. Ten micrograms of pCD7CAT were transfected into 8E51 cells as a positive control. Shown at the bottom is the percentage of relative CAT activity.

the polymeric-TAR inhibited Tat-induced HIV-1 LTR-CAT activity by 27.7-fold or 96% inhibition (Fig. 1B and Table 1A). Thus, polymeric-TAR decoy can inhibit both TNF and HIV-1 promoter activation induced by Tat. However, since these experiments were done by a transient transfection system, only some of the cells will take up at the same time all the plasmids. This may reduce the efficacy of the polymeric-TAR construct which in permanently expressing cells has been shown to inhibit up to 99% of viral replication (*41*).

We have previously shown that the TNFβ promoter is activated in cells constitutively expressing the first exon of Tat and in a cell line chronically infected with a *pol*-defective HIV-1 provirus (*25*). To determine whether polymeric-TAR could inhibit TNFβ activation in these systems, 5 μg of pTNFβCAT and 5 μg of either LTR-50 TAR or its control LTR-0 TAR were transfected into these two cell lines. For 8E51 cells, pCD7CAT was used as a positive control to demonstrate the endogenous expression of functional Tat protein. As shown in Fig. 2 and Table 1B, polymeric-TAR inhibited Tat-induced TNFβ gene expressions in both cell lines.

TABLE 1B

Inhibition of Tat-Mediated Activation of the TNFβ Promoter by Polymeric-TAR in Human CD4$^+$ T Cell Lines

Cell line	TNFβCAT activity (%)		Percentage of Inhibition
	LTR-0 TAR	LTR-50 TAR	
Jurkat + TAT	100	29 [22–34]	71 [66–78]
H9TAT 1	100	23 [3–45]	77 [55–97]
8E51	100	53 [9–70]	47 [30–91]
Jurkat + TAX	100	136 [96–171]	0

Note. pCVTAT (2 μg) or HTLV-TAX (2 μg) and pTNFβCAT (2 μg) were cotransfected with LTR-50 TAR (6 μg) or LTR-0 TAR (6 μg) into Jurkat cells; or pTNFβCAT (5 μg) and LTR-50 TAR (5 μg) or LTR-0 TAR (5 μg) were transfected into H9TAT 1 or 8E51 cells. CAT activity was determined as described previously (18). Percentage of relative TNFβCAT activity and percentage of inhibition by LTR-50 TAR was calculated on the level of CAT activity obtained with the control vector LTR-0 TAR. Shown are the average values and the number in brackets indicates the range of minimum and maximum values obtained in three to six repeated experiments for each cell type.

We have previously shown that polymeric-TAR does not affect Tax-induced expression of HTLV-I (*39*) and that Tax activates TNFβ gene expression by acting through the Sp1 and NFκB binding sites and does not require the TAR-like structure (*26, 49, 50*). To test whether inhibition by polymeric-TAR was specific for Tat-mediated TNFβ gene expression, the Tax expressing plasmid HTLV-Tax (*47*) was cotransfected with the pTNFβCAT and LTR-50 TAR or the control vector (LTR-0 TAR) into Jurkat cells. Although Tax did induce TNFβ gene expression, this activation was not inhibited by polymeric-TAR; in fact, the levels of TNFβ gene expression were the same in cells transfected with polymeric-TAR or with the vector alone (Fig. 3 and Table 1B), indicating that polymeric-TAR specifically interferes with Tat function.

These results reinforce our earlier work showing that Tat activates TNFβ gene expression through a TAR-like structure in both *tat*-transfected or HIV-1-infected CD4$^+$ T cells and indicate that polymeric-TAR decoys can specifically inhibit Tat-induced TNFβ gene expression. Particularly, the control with the Tax expressing construct is to demonstrate the different mechanism of transcriptional activation between Tat and Tax and therefore the

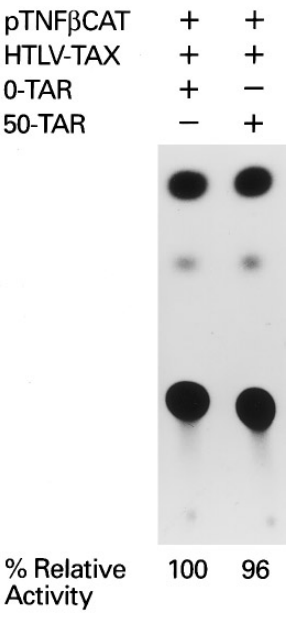

FIG. 3. Polymeric-TAR does not inhibit the activation of the TNFβ promoter induced by HTLV-I Tax. Representative CAT assay after co-transfection of pTNFβCAT (2 μg), HTLV-I-TAX (2 μg) with LTR-50 TAR (50 TAR) (6 μg), or LTR-0 TAR (0 TAR) (6 μg) into Jurkat cells. Shown at the bottom is the percentage of relative CAT activity.

specificity of the poly-TAR, since Tax transactivates the TNFβ promoter via SP1- and NFκB-mediated pathways and not via the TAR-like sequence. As shown in Fig. 3, poly-TAR does not inhibit the transactivation of TNFβ by Tax. It can also be interpretated that in the presence of Tax, 0-TAR and 50-TAR induce the same TNFβCAT activity, suggesting again that no double-stranded kinase activity is activated by poly-TAR. However, TNFβ gene expression mediated via physiological stimuli, through cellular transcription factors such as NFκB and SP1, will not be blocked by polymeric-TAR, suggesting a lack of toxicity *in vivo.* In addition, the polymeric-TAR expressing construct is under the control of the HIV-1 LTR; therefore, it is Tat-inducible. When Tat activity is inhibited by the polymeric-TAR construct, the expression of TAR RNA will also be decreased by the lower amount of Tat. Therefore, no excess amount of TAR will be synthesized. This is an obvious advantage for a gene therapy approach since it may avoid problems of toxicity and unwanted effects due to a constitutive expression. Since our previous studies have indicated that TNFβCAT activity well correlates with activation of endogenous TNF RNA or protein expression in HIV-1-infected cells or Tat-expressing cells (*25*), these results suggest that polymeric-TAR decoys can inhibit HIV-1 gene expression induced by Tat directly or indirectly through increased secretion of TNF. As TNF plays an important role in AIDS pathogenesis and associated disorders, a gene therapy approach using the polymeric-TAR gene which inhibits the activation of both HIV-1 and TNF gene expression may represent a useful approach for an effective therapy against AIDS.

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